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Morphologic analysis of intra-epidermic nerve fibers in small fiber neuropathy patients

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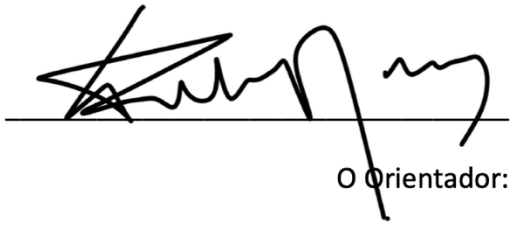
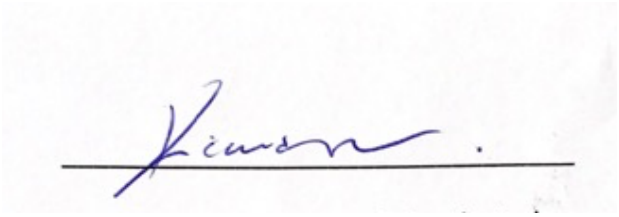


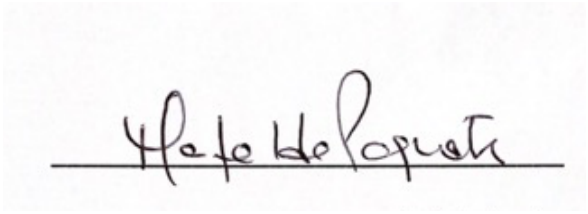
O Orientador:

(Luís Filipe Oliveira da Maia)



O Coorientador:

(Ricardo Jorge Ferreira Taipa)



A Estudante:

(Mafalda Isabel Almeida Paquete)

Porto, junho de 2022

À minha avó, Maria Odete.

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Análise morfológica das fibras nervosas intra-epidérmicas em doentes com neuropatia de pequenas fibras

Resumo

Introdução: As neuropatias de pequenas fibras são um grupo heterogéneo de doenças que afetam fibras Aδ mielinizadas e fibras C não mielinizadas. Os critérios diagnósticos para este grupo de patologias dividem-se em três grupos que incluem a avaliação clínica e o exame neurológico, os parâmetros neurofisiológicos e os parâmetros neuropatológicos, com a determinação da densidade fibras nervosas intra-epidérmicas.

No presente estudo, pretendeu-se, especificamente, explorar uma técnica semiautomática para a caracterização de fibras nervosas intra-epidérmicas de biópsias de pele, no que diz respeito à densidade e a características morfológicas adicionais, e correlacionar estas características com o grau gravidade da polineuropatia.

Materiais e Métodos: Amostras de biópsias de pele de um grupo pacientes com diferentes graus de Polineuropatia Amiloidótica por Transtirretina Hereditária foram coradas com produto de gene de proteína (PGP) 9.5, como marcador axonal e 4',6-diamidino-2-fenilindol (DAPI), como marcador nuclear, e fotografados usando um microscópio de fluorescência de campo amplo. Após a aquisição, as imagens foram preparadas e pré-processadas para análise posterior. As imagens foram divididas em dois canais diferentes: o canal DAPI, usado para a deteção da linha intradérmica e o canal PGP, utilizado para caracterizar e determinar os parâmetros morfológicos estudados. Obteve-se a densidade de fibras nervosas intra-epidérmicas e o seu gradiente entre biópsias proximais e distais, o número total de fibras, o número de fibras não ramificadas, o número, percentagem e densidade de fibras ramificadas, o número total de ramos, o número médio de ramos por fibra, o comprimento total da fibra, o comprimento médio das fibras, o comprimento dos ramos terminais e o seu valor médio por fibra.

Resultados: Um grupo de onze portadores da mutação Val30Met do gene da transtirretina foram selecionados e incluídos neste estudo. A amostra foi dividida em dois grupos, de acordo com a avaliação clínica, exame neurológico e exames diagnósticos realizados. No grupo de participantes sem polineuropatia (n=6) incluímos os pacientes assintomáticos (n=3) e paucissintomáticos (n=3), que se apresentavam sem alterações ao exame neurológico e com eletromiografia normal. O grupo dos participantes com polineuropatia conhecida (n=5) corresponde a todos os doentes com um quadro clínico compatível com uma polineuropatia de pequenas fibras e que se apresentam com

sintomas, alterações no exame neurológico e achados na eletromiografia concordantes com o diagnostico.

Para o grupo de doentes sem neuropatia, a média das densidades das fibras nervosas intra-epidérmicas foi de 13,26 $\pm$ 5,32 fibras/mm e 8,83 $\pm$ 4,60 fibras/mm, nas biópsias proximais e distais, respetivamente, com um gradiente entre estes valores de aproximadamente 35%.

Já para o grupo de doentes com neuropatia, a densidade média foi de 7,48 $\pm$ 3,28 fibras/mm nas biópsias proximais e 3,62 $\pm$ 2,64 fibras/mm nas biópsias distais, com um gradiente de 55%. Embora estes valores não apresentem diferenças estatisticamente significativas, o grupo de doentes sem neuropatia apresentou sempre valores mais próximos dos valores de referência.

No que diz respeito à comparação entre o grupo de doentes sem neuropatia e o grupo de doentes com neuropatia, concluímos que o número total de fibras (p = 0,030), o comprimento total das fibras (p = 0,030), o número (p = 0,017) e percentagem (p = 0,009) de fibras ramificadas e o número total de ramos (p = 0,048) apresentaram diferenças estatisticamente significativas entre os grupos, sempre com valores maiores para o grupo de doentes sem neuropatia.

Em relação à comparação entre os subgrupos, portadores assintomáticos e pacientes paucissintomáticos, não foi descrita, para as variáveis estudadas, nenhuma diferença estatisticamente significativa.

Discussão e Conclusão:

De acordo com a literatura e pela experiência prática relatada por médicos neurologistas, por vezes, os dados relativos à densidade de fibras nervosas intra-epidérmicas não são, por si só, suficientes para o diagnóstico e estratificação dos doentes com neuropatias de pequenas fibras.

Com este estudo exploratório, concluímos que a metodologia utilizada é válida e pode ser aplicada a outras amostras de pacientes, considerando esta ou outras patologias.

Além disso, constatámos que informações relacionadas com algumas características morfológicas (número total de fibras, comprimento total das fibras, número e percentagem de fibras ramificadas e número total de ramificações) se relacionam com o grau de severidade da doença.

Esperamos que, com este estudo, sejam consideradas outras características neuropatológicas, que juntamente com a densidade da fibras nervosas intra-epidérmicas, permitam uma melhor estratificação dos doentes segundo o grau de severidade da doença, que se possam, através delas, identificar os pacientes em estados mais precoces da evolução da doença, e, com isto, redefinir opções terapêuticas e melhor preservar o estado funcional do paciente.

Palavras-Chave: Neuropatia de pequenas fibras, Densidade de fibras intra-epidérmicas, Análise morfológica, Biopsia de pele

Morphologic analysis of intra-epidermic nerve fibers in small fiber neuropathy patients

Abstract

Introduction: Small fiber neuropathies are a heterogeneous group of disorders affecting myelinated Aδ-fibers and unmyelinated C-fibers. The diagnostic criteria for this group of pathologies are divided into three groups that include clinical assessment and neurological examination, neurophysiological parameters, and neuropathological parameters, including the determination of intraepidermal nerve fiber density.

In the present study we specifically aimed to explore a semi-automatized technique for the characterization of intra-epidermic nerve fibers from a skin biopsy in terms of fiber density and additional morphological features, and to correlate these nerve fiber features with the degree of severity of the polyneuropathy.

Materials and Methods: Skin biopsy specimens from a pool of patients with different degrees of polyneuropathy due to hereditary transthyretin amyloidosis were stained with the axonal marker of protein-gene-product (PGP) 9.5 and the nuclei marker of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and imaged using a widefield fluorescence microscope. After microscope acquisition, images were prepared and preprocessed for further analysis. Images were split into two different channels: the DAPI channel, used for the Intradermal line detection and the PGP channel, used to characterize, and determine the parameters mentioned above. Intraepidermal fiber nerve density and its gradient between proximal and distal biopsies, total number of fibers, number of non-branched fibers, number, percentage and density of branched fibers, total number of branches, number of branches per fiber, total and terminal branches length and total and terminal branches length per fiber were obtained.

Results: Eleven patients known to carry the transthyretin gene mutation Val30Met were selected and included in this study. The sample was dived into two groups, according to their history and clinical examination and diagnostic tests performed. In ATTR Patients without Neuropathy Group (n=6), we included asymptomatic (n=3) and paucisymptomatic patients (n=6), who did not present alterations in neurological examination or electromyography.

The ATTR Patients with Neuropathy Group (n=5) corresponds to all patients who already have a clinical condition compatible with small fiber polyneuropathy, presenting with symptoms, alterations in the neurological examination and electromyography findings congruent with the diagnosis.

For ATTR Patients without Neuropathy Group, the mean intraepidermal nerve fiber densities were 13,26 ± 5,32 fibers/mm and 8,83 ± 4,60 fibers/mm, in proximal and distal biopsies, respectively, with a gradient between these values of approximately 35%. For ATTR Patients with Neuropathy Group, the mean intraepidermal nerve fiber densities were 7,48 ± 3,28 fibers/mm in proximal biopsies, and 3,62 ± 2,64 fibers/mm in distal biopsies, with a gradient of approximately 55%. Although these values do not show statistically significant differences, the ATTR Patients without Neuropathy Group always presented values closer to the reference values.

When comparing ATTR Patients without Neuropathy Group and ATTR Patients with Neuropathy Group, we concluded that the total number of fibers (p = 0,030), the total branches length (p=0,030), the number (p = 0,017) and percentage (p = 0,009) of branched fibers and the total number of branches (p=0,048) showed statistically significant differences between the groups, always with higher values for ATTR Patients without Neuropathy Group.

Concerning the asymptomatic carriers and the paucisymptomatic patients, no statistically significant difference was described between these subgroups, for the variables under study.

Discussion and Conclusion: According to the literature and practical experience reported by neurologists, sometimes the intraepidermal fiber nerve density data alone are insufficient for the diagnosis and stratification of patients.

With this exploratory study, not only we conclude that the methodology used is valid and can be applied to other patient samples, considering this or other pathologies, but also, we found that some morphological characteristics are related to the degree of disease severity.

We hope that, with this study, other neuropathological characteristics will be considered and, along with the intraepidermal fiber nerve density data, allow a better stratification of patients according to the degree of severity of the disease. This set parameters can be used to identify patients in earlier stages of the disease's evolution and redefine therapeutic options to better preserve the patient's functional status.

Key Words: Small fiber neuropathies, Intraepidermal nerve fiber density, Morphologic analysis, Skin biopsy.

Abbreviation list

ATTR-PN	Transthyretin Amyloid Polyneuropathy
BL	Branch Length
CHUPorto	Centro Hospitalar Universitário do Porto
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
IDL	Intradermal Line
IENFD	Intraepidermal Nerve Fiber Density
ML	Manual Intradermal Line
PGP	Protein-gene-product
SFN	Small Fiber Neuropathy
TBL	Terminal Branch Length

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Introduction

Polyneuropathy is a specific term that refers to a generalized, relatively homogeneous process affecting multiple peripheral nerves, usually simultaneously, with the distal nerves usually affected most prominently.[1] Although it is assumed that polyneuropathy affects a considerable proportion of the population, the exact prevalence and incidence of the disease are not well known.[2] Regardless of the wide range of severity levels, it is known that the overall impact on a person's quality of life is notorious.[3]

Over 100 different causes of polyneuropathy have been identified, with diabetes as most important risk factor.[2] They can be distinguished in acquired or, less frequently, hereditary.

The most common acquired triggers are listed in Table I.[1,4, 5]

Concerning hereditary causes, it is worth noting Marie-Tooth Charcot Disease and Familial Amyloidotic Polyneuropathy.[6]

Patients with polyneuropathy characteristically present with paresthesia. The onset is initially distal, more intense during the night, waking the patient several times. In addition, patients tend to lose the sensation of vibration and the Achilles reflexes are reduced. A burning sensation, weakness, and tightness in the feet, as if they "had socks on", is also often described.[7-9]

As the pathology progresses, patients report paresis, initially, of the short extensors of the toes that progresses to the long extensors. Later, sensory changes and muscle weakness also extend to the upper limbs.[7-9]

Small fiber neuropathies (SFNs) are a type of peripheral polyneuropathy affecting myelinated Aδ-fibers and unmyelinated C-fibers.[10] Characterized as a heterogeneous group of disorders, SFN affects 0,1% of the general population,[11] and has an overall severe impact on quality of life, both physically and mentally.[12] It is associated with various disorders, such as metabolic (diabetes mellitus), infectious (human immunodeficiency virus), inflammatory (Sjogren's syndrome), and genetic (Fabry disease) diseases, but the cause is often idiopathic.[12]

One of the causes for SFNs are degenerative diseases as Transthyretin Amyloidosis Polyneuropathy (ATTR-PN). ATTR-PN is an autosomal dominant disease due to mutations in transthyretin (TTR) gene. The accumulation of abnormal proteins in nervous tissues results in the development of a progressive axonal polyneuropathy. The most common mutation is the TTR Val30Met, which is highly frequent in Portugal.[13]

Typically, clinical presentation of SFN is that of a symmetrical, length-dependent polyneuropathy associated with sensory or autonomic symptoms.[14,15]

Beyond history and clinical examination, the diagnostic tests to identify SFN include electromyography, quantitative sensory testing and intra-epidermic nerve fiber quantification.[16-19] Evaluating skin nerve fibers is a valuable method to diagnose SFN and we have recently improved procedure through an automated method to determine Intraepidermal Nerve Fiber density (IENFD) in skin nerve fibers.[11]

However, more fiber morphological parameters can be used to better characterize the onset of polyneuropathy and group patients more realistically.

In the present study we specifically aimed to explore the possibility to extend the intra-epidermal nerve fiber counting in terms of morphological characteristics. We used a genetic determined form of neuropathy, namely ATTR, to correlate these nerve fiber features with the degree of severity of the polyneuropathy.

We use advanced microscopy techniques to prepare and preprocess images for further analysis. Intraepidermal fiber nerve density and its gradient between proximal and distal biopsies, total number of fibers, number of non-branched fibers, number, percentage and density of branched fibers, total number of branches, number of branches per fiber, total and terminal branches length and total and terminal branches length per fiber were obtained.

After completing this study, we expect to have defined a set of parameters that are associated with different disease stages and patient functional status. If successful, these neuropathological features may improve SFN patient stratification for treatments and may constitute clinical trial endpoints.

Materials and methods

Patients

Eleven patients, under follow-up at Centro Hospitalar Universitário do Porto (CHUPorto), known to carry the transthyretin gene mutation Val30Met were selected and included in this study.

The sample was dived into two groups, according to their history and clinical examination and diagnostic tests performed. In ATTR Patients without Neuropathy Group (n=6), we included Asymptomatic Carriers (n=3) and Paucisymptomatic Patients (n=6), who did not present alterations in neurological examination or electromyography.

The ATTR Patients with Neuropathy Group (n=5) corresponds to all patients who already have a clinical condition compatible with small fiber polyneuropathy, presenting with symptoms, alterations in the neurological examination and electromyography findings congruent with the diagnosis.

Proximal and distal skin biopsies, that has already been performed within an approved project [(180-18 (155-DEFI/154-CES)], were selected and included in this study.

All participants gave their written informed consent for the previous study, which was approved by the local Ethics Committee of CHUPorto (PT) in accordance with the Helsinki Declaration.

Skin Biopsy and Staining

We followed the protocol implemented in the scientific article "Advantages of an Automated Method Compared with Manual Methods for the Quantification of Intraepidermal Nerve Fiber in Skin Biopsy" by the authors Corrà MF, et al..[11] Specifically:

Skin specimens were taken with a disposable 5-mm circular punch under sterile technique after topical anesthesia with lidocaine and no suture was needed. The anatomical sites of skin biopsies were the proximal thigh (20 cm below the greater trochanter) and the lateral side of the distal leg (10 cm above the malleolus) (Figure 1).

After fixation in 4% paraformaldehyde, specimens were incubated in 10% Saccharose at 4°C overnight, then frozen with 2-Methylbutan. Immunohistochemical labeling was performed on 50-µm frozen sections using rabbit polyclonal protein-gene-product (PGP) 9.5 antibody (Zytomed Systems, Berlin, Germany; 1:250). Indirect immunofluorescent technique with Cyanine 3 (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:50) as fluorescent secondary antibody was performed. The nuclei were stained with Vectashield antifade mounting medium with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Stained sections were stored at –20°C (Figure 1).

Biopsy Fluorescence Image Acquisition

We followed the protocol implemented in the scientific article "Advantages of an Automated Method Compared with Manual Methods for the Quantification of Intraepidermal Nerve Fiber in Skin Biopsy" by the authors Corrà MF, et al..[11] Specifically:

The same skin specimens were then imaged using a motorized widefield fluorescence microscope equipped with an HC PL FLUOTAR L 40x/0.60 objective (Leica DMI6000, Leica Microsystems). The nuclei were stained with DAPI (AT—Excitation: 340–380; BS: 400; Emission: 425 LP), and the rabbit polyclonal protein-gene-product 9.5 antibody coupled with the Cyanine 3 was used as a panaxonal marker (TX2 Excitation: 540–580; BS: 595; Emission: 607–683). A Z-stack was acquired with a step size of 695nm. The stack's upper and lower limits were defined to include all fibers from the epidermis and dermis. Images were acquired with a Hamamatsu Flash 4.2 sCMOS camera in mode binning 2 x 2, and the stitching was done within the LAS X Navigator extension (Figure 1).

Input Images

We followed the protocol implemented in the scientific article "Advantages of an Automated Method Compared with Manual Methods for the Quantification of Intraepidermal Nerve Fiber in Skin Biopsy" by the authors Corrà MF, et al..[11] Specifically:

After microscope acquisition, images are prepared and preprocessed for further analysis. Each image stack is merged to obtain the full skin section into a 4D mosaic image (XYZC). A scaling factor of 0.5 is applied to all images since the original image has a nonworkable size (~20Gb each). A maximum Z-projection of focus planes is applied to reduce z stacks into a 3D mosaic image (XYC). 8-Bit conversion and scale removal are done to always apply the same range of values in the following steps. Images should be oriented with the epidermal site in a right-down direction, so the user is prompted to correct image rotation if necessary. Images are split into 2 different channels: the DAPI channel is used for the Intradermal line detection and the PGP channel is used for the Intradermal line detection and the PGP channel to characterize and determine the morphological characteristics of the fibers under study (Figure 1).

Intradermal Line Detection and Fiber Tracing

A manual intradermal line (MIL) was drawn and measured, using Fiji drawing tools by following the epidermal cells stained in the DAPI channel. Fibers intersecting the MIL were manually drawed and automated counted using Fiji tool SNT, a framework for semi-automated tracing, quantitative analyses, and modeling of neuronal morphology.

Variables under Study

All the data collected were based on the electronic clinical files of the chosen patients using the "SClínico" computer system. For each patient, data related to sex and age were obtained for the construction of the demographic profile of the participants.

The morphological characteristics that lead to the variables under study are IEFND and its gradient between proximal and distal biopsies, total number of fibers, number of non-branched fibers, number, percentage and density of branched fibers, total number of branches (Figure 2), number of branches per fiber, total and terminal branches length (Figure 3 and Figure 4) and total and terminal branches length per fiber were obtained.

In each biopsy, the length of the intradermal line (IDL) (Figure 5) was measured and used to calculate some of the variables under study.

Statistical Analysis

The summary measures of the variables were presented according to their type and distribution.

All data was analyzed using Microsoft Excel, IBM SPSS Statistic version 26 platforms.

The test used to compare the variables under study was the Mann-Whitney U test with significance set at $p < 0,05$.

The statistical analysis was divided into two stages. First, we included all fibers, branched and non-branched fibers. In this phase the variables analyzed were Intraepidermal fiber nerve density and its gradient between proximal and distal biopsies, total number of fibers, number of non-branched fibers, number and percentage of branched fibers, total branches length and total branches length per fiber. All biopsies with 1 or more fibers were included.

During the second stage, we only included branched fibers. The variables evaluated were branched fiber density, number of branched fibers, total number of branches, number of branches per fiber, total and terminal branches length and total and terminal branches length per fiber. For this phase only sections with 1 or more branched fibers were included.

Regarding the variables related to the IENFD gradient between proximal and distal biopsies, for case #1 and case #7, in which the distal IENFD was higher than proximal IENFD, we imputed a value of 0 to the decrease between proximal and distal IENFD.

Results

Patients

A total of twenty-two skin biopsy specimens from a total of eleven participants with different degrees of polyneuropathy due to hereditary transthyretin amyloidosis (ATTR-PN) were analyzed. The mean age of the eleven subjects was 46,4 years old, with a minimum age of 31 years and a maximum of 71 years. Of the eleven participants, seven were women.

ATTR Patients without Neuropathy Group is composed of six participants, two of them male, with a mean age of approximately 43 years old. Three of these patients correspond to Asymptomatic Carriers Subgroup with a mean age of nearly 42 years old. The remaining three correspond to Paucisymptomatic Patients Subgroup, all female, with a mean age about 44 years old.

ATTR Patients with Neuropathy Group is composed of five participants, two of them male, with a mean age of approximately 50 years old. (Table II and Table III)

ATTR Patients without Neuropathy and ATTR Patients with Neuropathy

All Fibers Analysis

This sample includes all fibers from biopsies that contain at least 1 fiber.

For ATTR Patients without Neuropathy Group, the mean **intraepidermal nerve fiber densities** were 13,26 ± 5,32 fibers/mm and 8,83 ± 4,60 fibers/mm, in proximal and distal biopsies, respectively, with a **gradient** between these values of approximately 35%. For ATTR Patients with Neuropathy Group, the mean intraepidermal nerve fiber densities were 7,48 ± 3,28 fibers/mm in proximal biopsies, and 3,62 ± 2,64 fibers/mm in distal biopsies, with a gradient of approximately 55%. There were no statistically differences between groups regarding these variables. (Table IV)

Regarding the **number of fibers**, in the proximal biopsies, the mean ± standard deviation was 42,00 ± 15,96 fibers and 22,20 ± 7,98 fibers, for ATTR Patients without Neuropathy and ATTR Patients with Neuropathy groups, respectively. For the distal biopsies, the mean ± standard deviation was 28,83 ± 16,59 fibers for ATTR Patients without Neuropathy Group and 11,00 ± 7,40 fibers for ATTR Patients with Neuropathy Group. There was statistically difference between groups, with higher values in ATTR Patients without Neuropathy Group, for the proximal biopsies (p = 0,030). For the distal biopsies, there were no statistically differences (p = 0,052).

Considering the **total branch length**, in the proximal biopsies, the mean ± standard deviation was 2210,34 ± 894,29 µm, for ATTR Patients without Neuropathy Group and 1122,31 ± 546,32 µm, for ATTR Patients with Neuropathy Group. There was statistically difference between groups, with higher values in ATTR Patients without Neuropathy Group, (p = 0,030)

Respecting the **number of branched fibers**, in proximal biopsies, the mean ± standard deviation for ATTR Patients without Neuropathy and ATTR Patients with Neuropathy groups was, respectively, 12,33 ± 6,02 fibers and 5,80 ± 3,25 fibers. As for the distal biopsies, the average was 11,83 ± 6,41 fibers in ATTR Patients without Neuropathy Group and 2,40 ± 2,42 fibers in ATTR Patients with Neuropathy Group Although, there were no statistically differences between groups regarding the proximal biopsies (p = 0,052), in the distal sample this variable was statistically different between groups, with higher values in ATTR Patients without Neuropathy Group (p = 0,017).

Concerning the **percentage of branched fibers**, in the distal biopsies, the average was, in ATTR Patients without Neuropathy Group, 42,25 ± 8,54% and, in ATTR Patients with Neuropathy Group, 13,55 ± 13,50%. This variable was statistically different between groups with higher values in ATTR Patients without Neuropathy Group (p = 0,009).

Branched Fibers Analysis

This sample includes all branched fibers from biopsies that contain at least 1 fiber with branches. Distal biopsies from case #1 and case #8 are omitted as they do not fulfil this requirement.

Regarding the **total number of branches**, in the distal biopsies, the mean ± standard deviation was 36,00 ± 19,24 branches for ATTR Patients without Neuropathy Group and 10,33 ± 4,78 branches for ATTR Patients with Neuropathy Group. Although, there were no statistically differences between groups regarding the proximal biopsies, in the distal sample this variable was statistically different between groups, with higher values in ATTR Patients without Neuropathy Group (p = 0,048).

Although no other variable showed statistically significant differences between this groups, the descriptive analysis of the remaining variables under study is listed in Table IV, Table V, Table VI, Table VII and Table VIII.

ATTR asymptomatic carriers and ATTR paucisymptomatic patients

None of the variables under study showed statistically significant differences between this groups. Nonetheless, the descriptive analysis of the variables is listed in Table IX, Table X, Table XI, Table XII and Table XIII.

Discussion

In this study, we explore a new methodology and parameters for the study of intra-epidermic nerve fibers. Surprisingly, we did not found differences between the clinical more affected neuropathy patients and the asymptomatic and paucisymptomatic patients regarding IENFD.[20] However, there was tendency in towards lower density in clinical affected group.

The reduced number of the sample and lack representativeness of collected data could explain this result. Additionally, considering the fibers density in relation to normative values for the population, even the considered asymptomatic patients have already severe fiber loss. These results should be interpreted with these limitations, and bigger sample, with younger patients should be pursued.

As a new data in this study, we conclude that the total number of fibers, total branch length and the number and percentage of branched fibers and the total number of branches are related to the degree of disease severity, always assuming higher values for the ATTR Patients without Neuropathy Group. These variables could be important to explore in future clinicopathological studies as possible clinical disease onset biomarker.

We did not find a statistically differences regarding all the others morphological characteristics studied. It is possible that the morphological characteristics used as variables under study are not effectively related to the degree of severity of the disease. However, these results could be in part because the small and insufficient to draw statistical conclusions number of selected patients or because the selected patients was already at an advanced stage of the disease, which led us to miss the period where these pathophysiological changes take place.

Although the groups with ATTR asymptomatic carriers and ATTR paucisymptomatic patients are clinically distinguishable, no significant differences were found when fibers morphological characteristics were analyzed. The small number of the sample can also justify this result. Furthermore, there may be no actual differences in the analyzed variables, since none of the participants in these groups fulfil the neuropathy diagnostic criteria.

There is limited literature regarding characterization of proximal fibers in patients with polyneuropathies. From our study, we can infer that the process that affects fiber morphology may take place later in time to the proximal fibers, but that is, for the time being, speculative.

Some **limitations** need to be addressed. For a greater reliability in the outcomes obtained, and in order to possibly find new correlations between other variables under study and the degree of disease severity, it would be necessary to increase the **size of the sample**, in in terms of the number of participants and the number of analyzed sections per biopsy and compare the results of the different groups to a **control group** that would include non-carrier and symptom-free participants.

Besides, for a better and more realistic division between Asymptomatic Carriers and Paucisymptomatic Patients, **complementary exams** as quantitative sensory tests can be performed so the criteria on which this division is based are not as subjective as the description of symptoms by the patients.

Conclusions

Small fiber polyneuropathies are diagnosed by quantifying the patient's intraepidermal nerve fiber density. According to the literature and practical experience reported by neurologists, sometimes these data alone are insufficient for the diagnosis and stratification of patients. Therefore, in this exploratory study, we morphologically analyzed skin biopsies from a sample of eleven patients with ATTR-PN, to find out if there is other morphological characteristic that could be used as a parameter for a more realistic stratification of patients.

Although the statistical results were not significant for all variables, not only we conclude that the methodology used is valid and can be applied to other patient samples, considering this or other pathologies, but also, we found that some morphological characteristics are related to the degree of disease severity.

We hope that this study may open the way for further research work to set parameters that are, along with the IENFD, associated with different disease stages and patient functional status. These neuropathological features may improve SFN patient stratification for treatments and may constitute clinical trial endpoint. We expect that this may lead off to identify patients at pre- or early symptomatic stages of SFN and anticipate the treatment with disease modifying therapies.

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Appendix

I – Tables

Table I. Most Common Acquired Causes of Polyneuropathy

Endocrine	Diabetes Mellitus, Hypothyroidism
Metabolic	Kidney/Liver failure, B12 deficiency, Porphyria
Immune-mediated	Guillain-Barré Syndrome, Chronic Inflammatory Demyelinating Polyneuropathy Paraproteinemias, Anti-MAG Neuropathy
Infectious	HIV, Lyme disease (Borrelia), CMV, Syphilis, Leprosy, Diphtheria
Iatrogenic	Antiretrovirals, Chemotherapy, Other drugs (ethambutol, isoniazid)
Toxins	Heavy metals, Lead, Arsenic, Alcohol
Paraneoplastic	Lung carcinoma (small cell)

Table II. Demographic Profile: ATTR Patients without Neuropathy and ATTR Patients with Neuropathy

Demographic Profile			
	Total	**ATTR Patients without Neuropathy**	**ATTR Patients with Neuropathy**
	(n=11)	(n=6)	(n=5)
Male sex (%)	36,4	33,3	40,0
Age (n)	46,4 ± 10,8	43,0 ± 9,4	50,4 ± 10,9

Table III. ATTR Asymptomatic Carriers and AATR Paucisymptomatic Patients

Demographic Profile		
	ATTR Asymptomatic Carriers (n=3)	**ATTR Paucisymptomatic Patients** (n=3)
Male sex (%)	66,7	00,0
Age (n)	41,7 ± 9,0	44,3 ± 9,6

Table IV. ATTR Patients without Neuropathy and ATTR Patients with Neuropathy: IENFD and IENFD Gradient

Proximal Biopsies			
	ATTR Patients without Neuropathy	**ATTR Patients with Neuropathy**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
IENFD (n/mm)	13,26 ± 5,32	7,48 ± 3,28	0,082

Distal Biopsies			
	ATTR Patients without Neuropathy	**ATTR Patients with Neuropathy**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
IENFD (n/mm)	8,83 ± 4,60	3,62 ± 2,64	0,082

IENFD Gradient			
	ATTR Patients without Neuropathy	**ATTR Patients with Neuropathy**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
Gradient (%)	34,64 ± 31,49	54,65 ± 25,05	0,429

Table V. ATTR Patients without Neuropathy and ATTR Patients with Neuropathy: All Fibers Data - Proximal Biopsies

Proximal Biopsies			
	ATTR Patients without Neuropathy Mean ± Standard Deviation	**ATTR Patients with Neuropathy** Mean ± Standard Deviation	***p* value**
No. Fiber (n)	42,00 ± 15,96	22,20 ± 7,98	0,030
BL (µm)	2210,34 ± 894,29	1122,31 ± 546,32	0,030
BL/Fiber (µm/n)	53,00 ± 17,73	50,14 ± 16,61	0,931
No. Non-branched Fibers (n)	29,67 ± 14,11	16,40 ± 5,61	0,117
No. Branched Fibers (n)	12,33 ± 6,02	5,80 ± 3,25	0,052
% Branched Fibers (%)	29,49 ± 15,97	24,44 ± 9,35	0,792

Table VI. ATTR Patients without Neuropathy and ATTR Patients with Neuropathy: All Fibers Data - Distal Biopsies

Distal Biopsies			
	ATTR Patients without Neuropathy Mean ± Standard Deviation	**ATTR Patients with Neuropathy** Mean ± Standard Deviation	***p* value**
No. Fiber (n)	28,83 ± 16,59	11,00 ± 7,40	0,052
BL (µm)	1638,28 ± 1199,28	537,94 ± 407,39	0,126
BL/Fiber (µm/n)	54,17 ± 15,00	46,18 ± 6,67	0,247
No. Non-branched Fibers (n)	17,00 ± 10,68	8,60 ± 5,89	0,247
No. Branched Fibers (n)	11,83 ± 6,41	2,40 ± 2,42	0,017
% Branched Fibers (%)	42,25 ± 8,54	13,55 ± 13,50	0,009

Table VII. ATTR Patients without Neuropathy and ATTR Patients with Neuropathy: Branched Fibers Data - Proximal Biopsies

Proximal Biopsies			
	ATTR Patients without Neuropathy Mean ± Standard Deviation	**ATTR Patients with Neuropathy** Mean ± Standard Deviation	***p* value**
Branched Fibers Density (n/mm)	4,02 ± 2,27	2,89 ± 0,50	0,177
No. Branched Fibers (n)	12,33 ± 6,02	5,80 ± 3,25	0,052
Total No. Branches (n)	40,50 ± 25,60	17,80 ± 7,83	0,126
Branches/Fiber (n/fiber)	3,12 ± 0,49	3,46 ± 1,08	1,000
BL (µm)	1207,70 ± 740,62	517,76 ± 345,23	0,126
BL/Fiber (µm/n)	97,88 ± 21,05	87,38 ± 31,57	0,537
TBL (µm)	625,91 ± 382,89	342,22 ± 225,40	0,247
TBL/Fiber (µm /n)	53,43 ± 15,23	57,18 ± 20,49	1,000

Table VIII. ATTR Patients without Neuropathy and ATTR Patients with Neuropathy: Branched Fibers Data - Distal Biopsies

Distal Biopsies			
	ATTR Patients without Neuropathy Mean ± Standard Deviation	**ATTR Patients with Neuropathy** Mean ± Standard Deviation	***p* value**
Branched Fibers Density (n/mm)	3,61 ± 1,77	1,24 ± 0,94	0,167
No. Branched Fibers (n)	11,83 ± 6,41	3,67 ± 2,36	0,095
Total No. Branches (n)	36,00 ± 19,24	10,33 ± 4,78	0,048
Branches/Fiber (n/fiber)	3,04 ± 0,66	3,14 ± 0,65	1,000
BL (µm)	972,28 ± 687,68	333,33 ± 235,76	0,095
BL/Fiber (µm/n)	75,64 ± 23,59	87,45 ± 12,01	0,548
TBL (µm)	474,82 ± 377,39	154,52 ± 121,08	0,167
TBL/Fiber (µm /n)	35,81 ± 14,12	38,88 ± 12,94	1,000

Table IX. ATTR Asymptomatic Carriers and ATTR Paucisymptomatic Patients: IENFD Gradient

Proximal Biopsies			
	ATTR Asymptomatic Carriers	**ATTR Paucisymptomatic Patients**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
IENFD (n/mm)	12,88 ± 7,48	13,64 ± 0,61	0,700

Distal Biopsies			
	ATTR Asymptomatic Carriers	**ATTR Paucisymptomatic Patients**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
IENFD (n/mm)	9,83 ± 5,94	7,82 ± 2,26	1,000

IENFD Gradient			
	ATTR Asymptomatic Carriers	**ATTR Paucisymptomatic Patients**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
Gradient (%)	27,41 ± 38,76	41,87 ± 19,40	0,700

Table X. ATTR Asymptomatic Carriers and ATTR Paucisymptomatic Patients: All Fiber Data - Proximal Biopsies

Proximal Biopsies			
	ATTR Asymptomatic Carriers Mean ± Standard Deviation	**ATTR Paucisymptomatic Patients** Mean ± Standard Deviation	***p* value**
No. Fiber (n)	42,33 ± 22,07	41,67 ± 4,71	1,000
BL (µm)	2020,05 ± 1099,27	2400,63 ± 564,52	1,000
BL/Fiber (µm/n)	45,88 ± 6,27	60,11 ± 22,09	1,000
No. Non-branched Fibers (n)	33,00 ± 16,87	26,33 ± 9,57	1,000
No. Branched Fibers (n)	9,33 ± 5,44	15,33 ± 4,99	0,700
% Branched Fibers (%)	20,25 ± 5,91	38,73 ± 17,44	0,200

Table XI. ATTR Asymptomatic Carriers and ATTR Paucisymptomatic Patients: All Fiber Data - Distal Biopsies

Distal Biopsies			
	ATTR Asymptomatic Carriers Mean ± Standard Deviation	**ATTR Paucisymptomatic Patients** Mean ± Standard Deviation	***p* value**
No. Fiber (n)	32,33 ± 21,30	25,33 ± 8,50	1,000
BL (µm)	1879,47 ± 1484,63	1397,10 ± 745,71	1,000
BL/Fiber (µm/n)	56,71 ± 18,06	51,64 ± 10,53	1,000
No. Non-branched Fibers (n)	20,33 ± 13,82	13,67 ± 3,86	1,000
No. Branched Fibers (n)	12,00 ± 7,79	11,67 ± 4,64	0,700
% Branched Fibers (%)	39,41 ± 10,97	45,09 ± 3,06	0,700

Table XII. ATTR Asymptomatic Carriers and ATTR Paucisymptomatic Patients: Branched Fibers Data - Proximal Biopsies

Proximal Biopsies			
	ATTR Asymptomatic Carriers Mean ± Standard Deviation	**ATTR Paucisymptomatic Patients** Mean ± Standard Deviation	***p* value**
Branched Fibers Density (n/mm)	2,85 ± 1,77	5,19 ± 2,10	0,700
No. Branched Fibers (n)	9,33 ± 5,44	15,33 ± 4,99	0,700
Total Branches (n)	29,00 ± 17,38	52,00 ± 27,29	0,700
Branches/Fiber (n/fiber)	3,07 ± 0,09	3,17 ± 0,68	1,000
BL (µm)	980,15 ± 605,44	1435,26 ± 791,79	0,700
BL/Fiber (µm/n)	108,77 ± 15,29	86,98 ± 20,38	0,200
TBL (µm)	451,26 ± 212,16	800,55 ± 432,65	0,700
TBL/Fiber (µm /n)	58,14 ± 17,42	48,72 ± 10,77	1,000

Table XIII. ATTR Asymptomatic Carriers and ATTR Paucisymptomatic Patients: Branched Fibers Data – Distal Biopsies

Distal Biopsies			
	ATTR Asymptomatic Carriers	**ATTR Paucisymptomatic Patients**	***p* value**
	Mean ± Standard Deviation	Mean ± Standard Deviation	
Branched Fibers Density (n/mm)	3,62 ± 2,15	3,59 ± 1,27	0,700
No. Branched Fibers (n)	12,00 ± 7,79	11,67 ± 4,64	0,700
Total Branches (n)	35,67 ± 21,75	36,33 ± 16,36	1,000
Branches/Fiber (n/fiber)	3,05 ± 0,82	3,03 ± 0,45	1,000
BL (µm)	1057,27 ± 789,59	887,29 ± 554,90	1,000
BL/Fiber (µm/n)	82,36 ± 25,65	68,92 ± 19,10	0,400
TBL (µm)	560,19 ± 451,65	389,45 ± 257,46	0,700
TBL/Fiber (µm /n)	41,85 ± 15,26	29,77 ± 9,66	0,400

2 – Figures

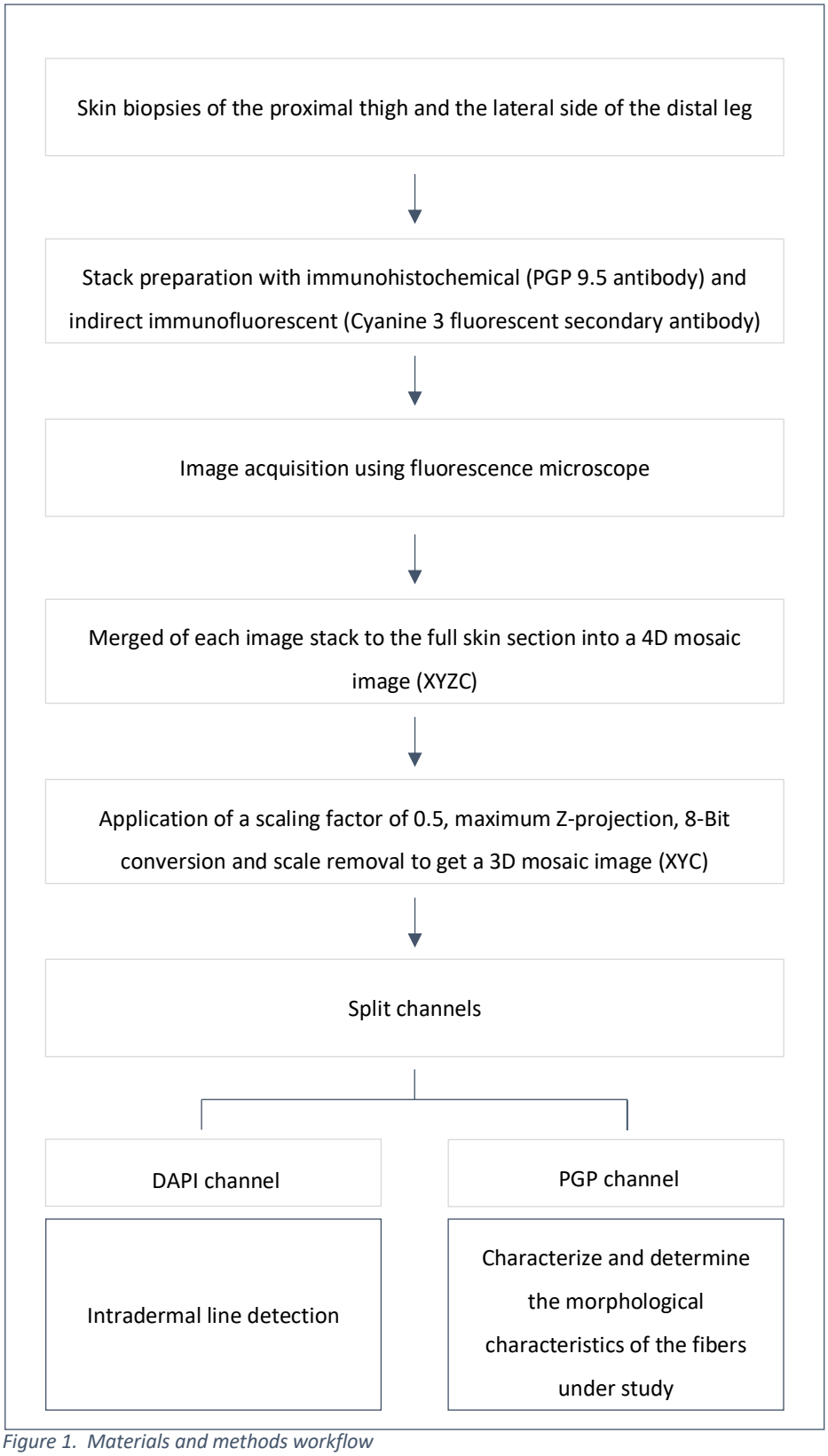

Figure 1. Materials and methods workflow

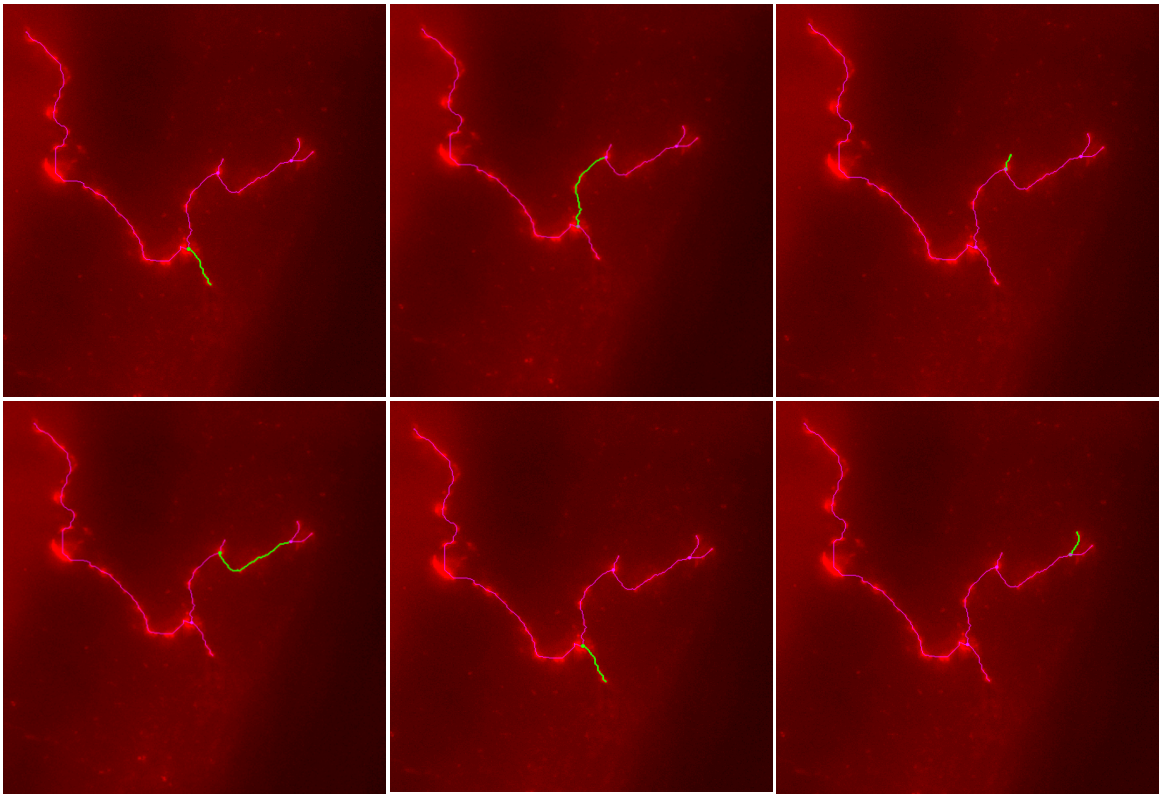

Figure 2. Total Number of Branches

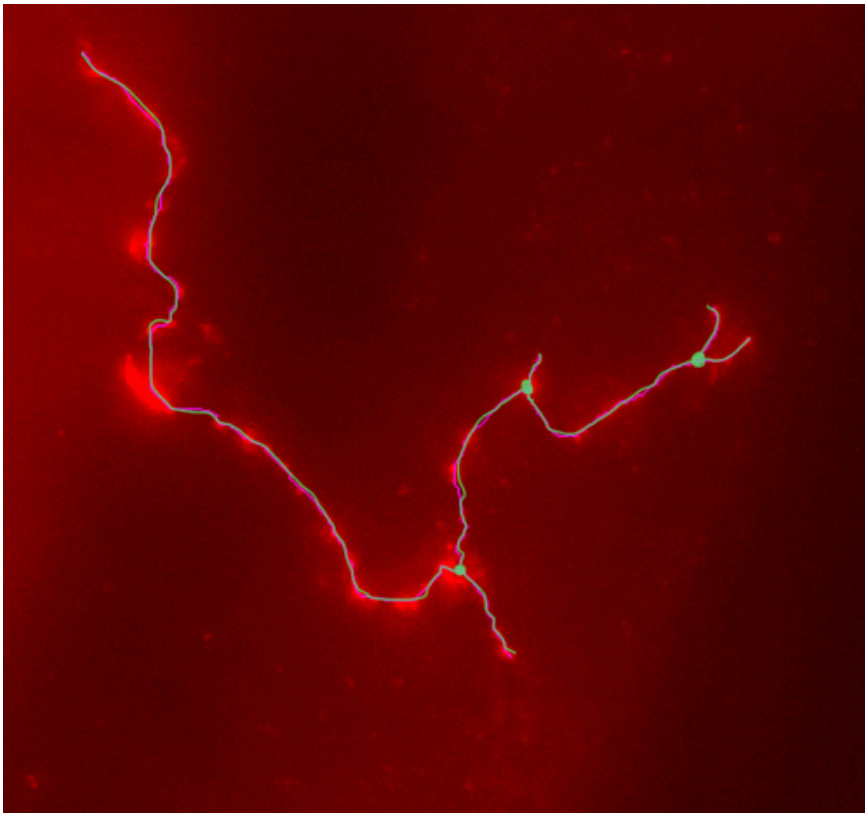

Figure 3. Total Branch Length

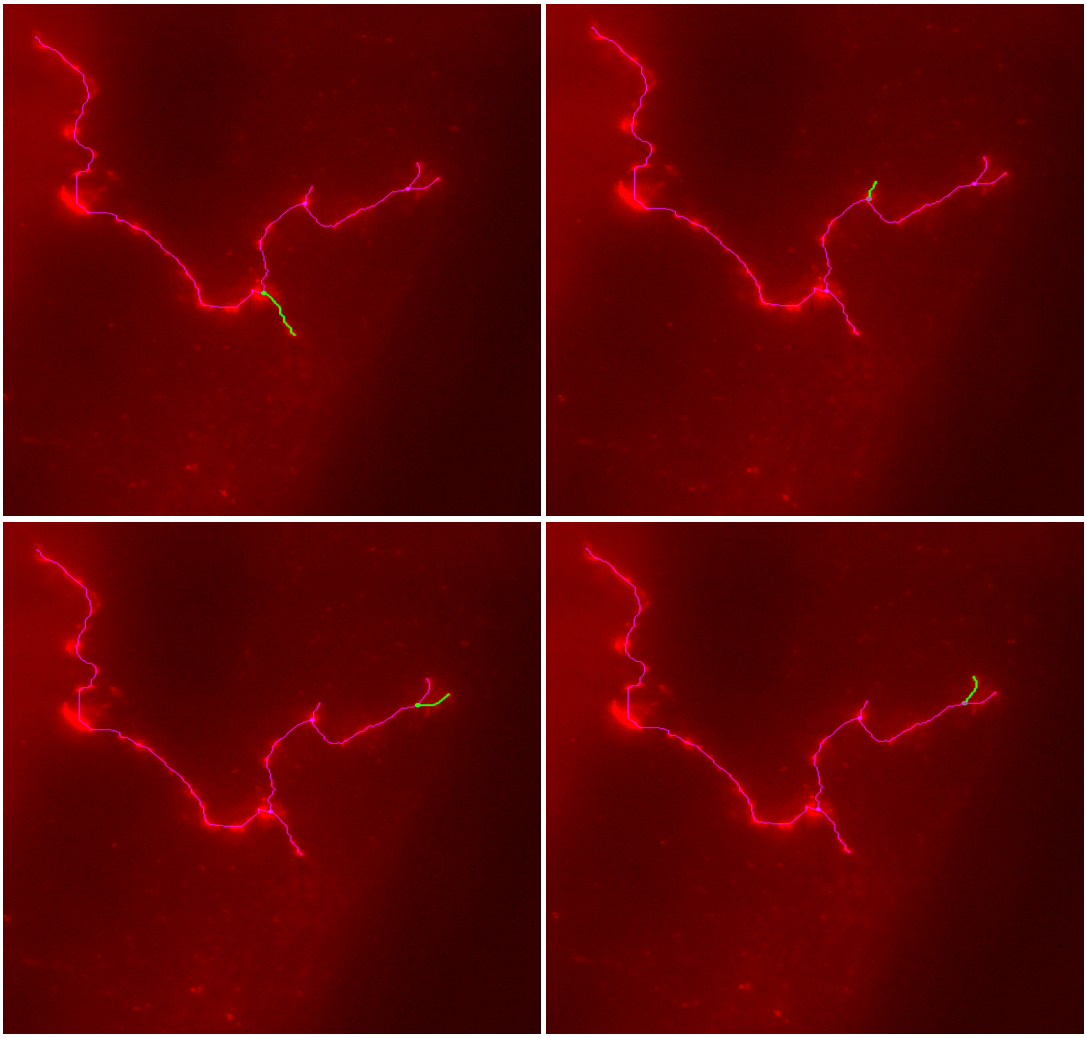

Figure 4. Terminal Branch Length

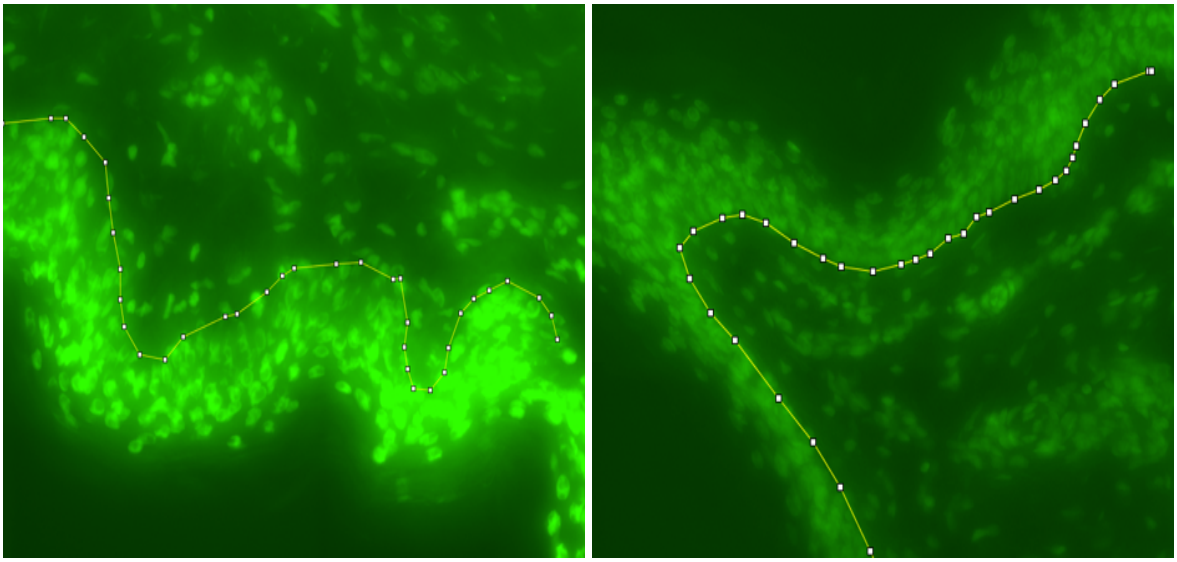

Figure 5. Manual Intradermal Line Detection on DAPI Channel